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Distribution of APGWa-immunoreactive Substances in the Central Nervous System and Reproductive Apparatus of *Helix aspersa*

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ABSTRACT—The distribution of substances related to the tetrapeptide APGWa was investigated in the central nervous system (CNS) and the reproductive apparatus of *Helix aspersa* by immunocytochemistry. In the CNS, APGWa immunoreactive neurons were detected in all ganglia except the pedal ganglia. Concerning the mesocerebrum of the cerebral ganglia, only neurons of the right mesocerebral lobe reacted positively to the antiserum. In the genital apparatus, positive neurons fibres were seen in the muscular layer of the penis and, in the gonad, an immunoreactive material occurred on the heads of some spermatozoa.

On the basis of these observations and of previous electrophysiological studies, an implication of AGPWa-like peptides in the control of mating behaviour is proposed. The significance of the positive reaction of the spermatozoa remains unclear.

INTRODUCTION

During the last ten years, many neuropeptides have been isolated and sequenced from invertebrates. Among these, the tetrapeptide APGWa was isolated and purified from the ganglia of the mollusc *Fusinus ferrugineus* [1]. At the same time, Smit *et al.* [2] were able to clone a gene from the anterior lobe of the cerebral ganglia of *Lymnaea stagnalis* which codes for APGWa. This peptide exerts a biological action on various molluscan muscles [3]; in *Lymnaea stagnalis* for instance, it is supposed to play a role in the control of the penial complex, especially during copulation [2, 4].

In order to collect some informations on the control of reproduction and sexual behaviour in *Helix aspersa,* we have undertaken immunocytochemical studies using antibodies directed against biologically active neuropeptides such as α-caudo-dorsal cell peptide (αCDCP), a peptide encoded by the genes of the egg-laying hormone of *Lymnaea stagnalis* (unpublished results). In the present work, we investigated for the occurrence of APGWa-like substances in the central nervous system and the genital apparatus. The nature of the factors which control the male copulatory behaviour in *Helix* is not known as yet but some morphological and physiological observations suggest that the right mesocerebrum is associated with a sexual function(s). Thus a special attention was paid to this part of the brain.

MATERIALS AND METHODS

Animals

Adult snails (*Helix aspersa*), bred in the Centre Universitaire d'Héliciculture* under controlled conditions (photoperiod: 18 h light 6 h dark, temperature: 20°C, humidity: 100%) were used. Three were sacrificed during copulation, three other were in an active non-reproductive state.

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Methods

Central nervous systems (CNS) and different parts of genital apparatus (ovotestis, sperm-oviduct, prostate, penis, dart pouch and multifid glands) were fixed in Bouin-Hollande fixative (+10% mercuric chloride) for 24 hr at room temperature. They were embedded in paraffin and serially sectioned at 6 μm.

Antibodies to APGWa were raised in rabbits. They were prepared and tested for specificity in the Department of Biology of the free University of Amsterdam according to a previously described procedure [6]. For immunocytochemical observations, the sections were processed with the PAP method [7]. After suppression of possible endogenous peroxidase activity by a rinse in phosphate buffer saline (PBS) containing 0.0125% H_2O_2, sections were incubated overnight with the primary antiserum (anti-APGWa, dilution 1/1000; 4°C). They were then treated with the second antiserum (goat anti-rabbit, dilution 1/200, 1 hr at room temperature) followed by PAP solution (dilution 1/100; 1 hr at room temperature). The peroxidase was visualized with 0.05% 3,3′ diaminobenzidine tetrahydrochloride in PBS containing 0.01% H_2O_2.

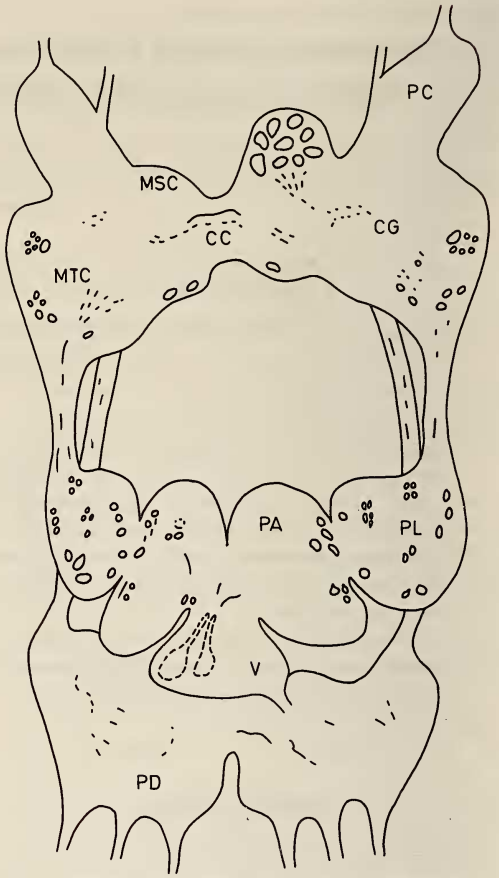


Fig. 1. Diagram of the central nervous system of *Helix aspersa* showing APGWa-immunoreactive perikarya (irregular circles) and fibres (short lines).
CC, cerebral commissure; CG, cerebral ganglia; PA, parietal ganglia; PD, pedal ganglia; PL, pleural ganglia; V, visceral ganglion; MSC, mesocerebrum; MTC, metacerebrum; PC, procerebrum.

RESULTS

Central nervous system

Immunoreactive cells were found in all ganglia except the pedal ganglia (Fig. 1). Depending on the animals, weak differences were observed for example in the intensity of staining or in the number of immunoreactive cells but they do not seem to be correlated with the mating or non-

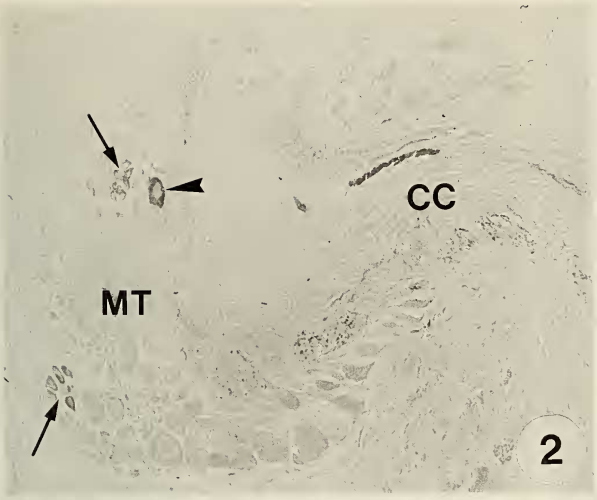
Fig. 2. Immunoreactive perikarya in the left cerebral ganglion. Two groups of small neurons (arrows) and one bigger cell (arrowhead) are located at the external margin. Note the presence of a large positive fibre in the cerebral commissure (CC). ×120. MT, metacerebrum.

Fig. 3. Section through the cerebral ganglia (CG). The neurons located in the right mesocerebral lobe exhibit a positive reaction (arrow) whereas those of the left lobe (arrowhead) are not stained. ×80. CC, cerebral commissure; DB, dorsal body area.

Fig. 4. Right cerebral ganglion. The axons (arrowheads) of the immunoreactive neurons (arrow) of the mesocerebral lobe project toward the right interganglionic connectives. ×100. MS, mesocerebrum; MT, metacerebrum; PC, procerebrum.

Fig. 5. Right parietal ganglion (PA). Note the presence of a group of positive cells (arrow) at the posterior margin, close to the pleural ganglion (PL). ×120. n, neuropil.

Fig. 6. Small strongly labelled perikarya (arrows) in the left pleural ganglion (PL). Immunoreactive fibres are visible in the cerebro-pleural connective (arrowhead). ×100.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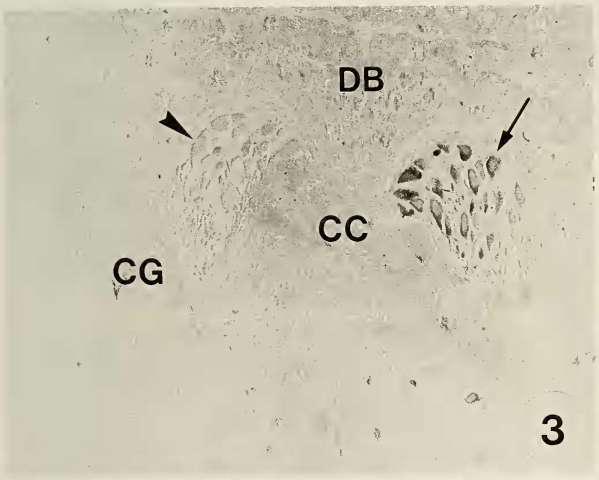
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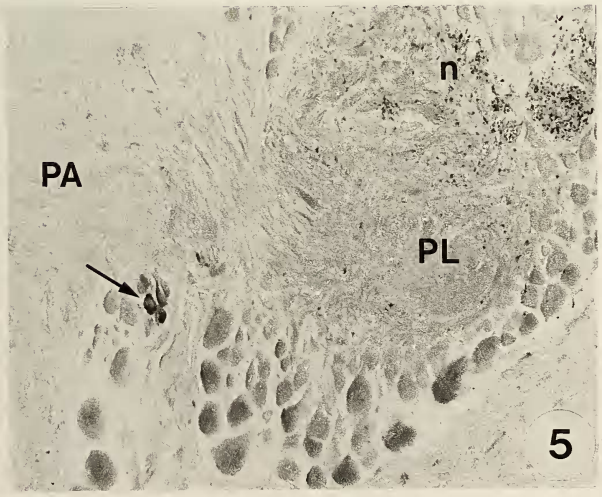
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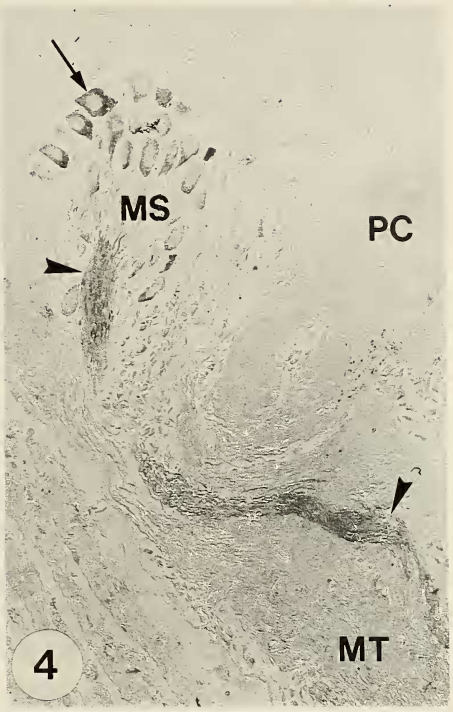
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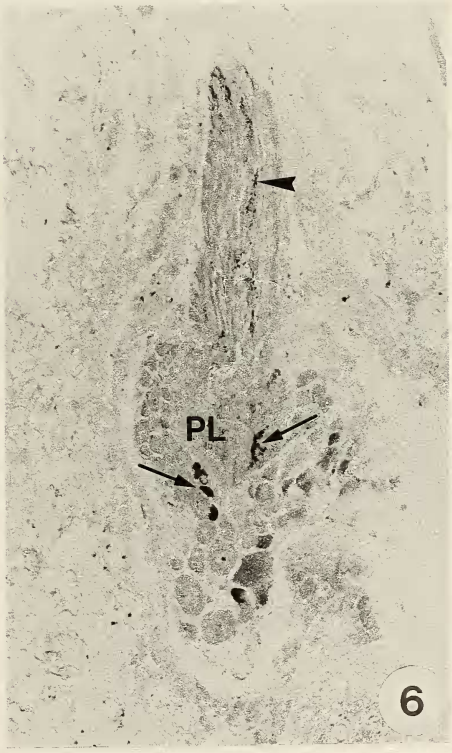
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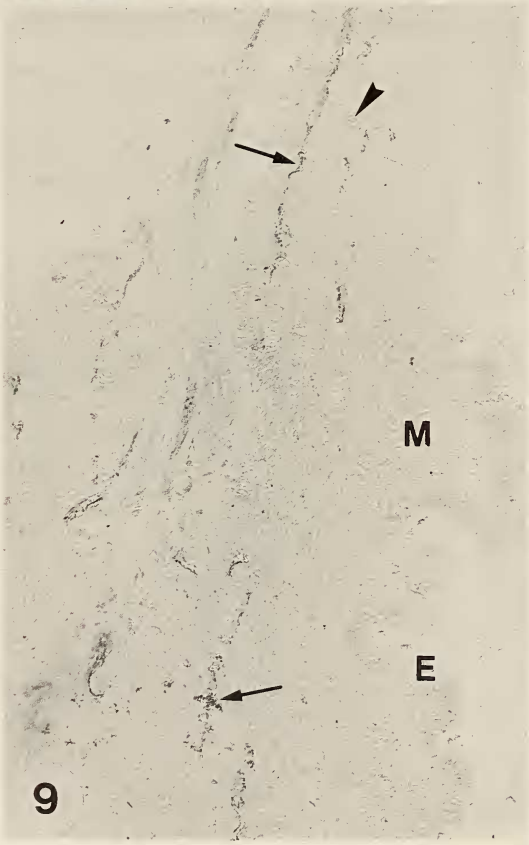
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mating state.

Cerebral ganglia: Three major groups of positive cells could be distinguished in each cerebral ganglion. Two cell groups were located at the lateral external margin of the metacerebrum (Fig. 1). The first group consisted of about 20 small cells (10 μm in diameter) and 1 larger cell (diameter= 25–30 μm) (Fig. 2). The second group, located posteriorly, consisted of about 15 cells measuring 20 μm in diameter (Fig. 2). The third group was exclusively found in the anterior lobe of the right mesocerebrum only (Figs. 3, 4). Among the population of pear-shaped cells (40–45 μm in diameter) characteristic of this area, 40 to 60 showed a positive reaction; their perikarya exhibited a less intense staining than those of the other groups; their axons formed a bundle running across the right cerebral ganglion to the origin of the cerebro-pleural and pedal connectives (Fig. 4); from this area, it was difficult to follow the axonal projections, most of them apparently ran through the right cerebro-pedal connective to the pedal ganglia. The right cerebro-pedal and cerebro-pleural connectives contained many more fibres than those on the left side. Positive fibres were present in the neuropil of each cerebral ganglion and large axons crossing the commissure were found (Fig. 2). Furthermore, immunoreactive fibres were found in some nerve roots such as the labial, penial and peritentacular nerves.

Parietal ganglia: About 10 cells of 25–30 μm in diameter in the anterior part of the ganglia, close to the pleural ganglia showed a positive reaction. At the posterior part, 7 elongated cells (20×10 μm) and 2 larger cells (30 μm) were found, occupying similar positions near the pleural ganglia (Fig. 5). In addition, a cluster of 7 to 10 darkly stained cells (15 μm in diameter) were located in the left parietal ganglion close to the anterior margin of the visceral ganglion. Several other positive perikarya were occasionally dispersed in the anterior half of the left parietal ganglion. A network of immunoreactive varicose fibres could be observed in the neuropils.

Pleural ganglia: Most of the positive cells were located at the lateral external side of the ganglia, 16 cells (12–15 μm) at the anterior part and about 15 cells (diameter 25 μm) posteriorly. In each ganglion a cluster of 40 small, strongly labelled, neurons (10–12 μm) occurred at the basis of the cerebro-pleural connective (Fig. 6). Another group consisting of about 40 intensely stained cells (10–15 μm) was observed medially on the anterior face of the ganglia (Fig. 7). About 12 additional cells of 15 μm in diameter were scattered in each ganglion and 2 neurons (25–30 μm) were visible posteriorly, near the parietal ganglia. Prominent bundles of immunoreactive fibres were observed in the neuropils (Fig. 7).

Visceral ganglion: 2 or 3 large neurons (140 μm) located at the posterior face stained moderately (Fig. 8). Positive fibres were revealed in the neuropil and in the nerve roots.

Pedal ganglia: Although no immunoreactive perikarya were found, many positive fibres were present in the neuropils, running in different directions towards the connectives and the nerves leaving these ganglia. Many more fibres were present in the right ganglion than in the left.

Genital apparatus

Among the investigated organs, immunoreactivity was only found in the penis and the gonad. In the penis, positive fibres were relatively numerous, especially in the proximal half. Thick tracts of varicose and non-varicose fibres, usually oriented in a longitudinal direction, ran in the well-developed muscular layer (Fig. 9). Small lightly stained peripheral neurons were seen occasionally (Fig. 9). In some gonads, a clear label occurred on

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Fig. 7. Cluster of strongly stained neurons (arrow) in the medial part of the right pleural ganglion. ×290. Arrowheads: positive fibres in the neuropil.

Fig. 8. Visceral ganglion (VG). Note the presence of a large neuron whose perikaryon and axon are moderately stained (arrow). ×120. n, neuropil.

Fig. 9. Longitudinal section of the penis. Positive fibres (arrows) are observed in the muscular layer (M). A small lightly stained cell is visible (arrowhead). ×120. E, epithelial layer.

Fig. 10. Gonadal acini. Note the strong reaction (arrows) on the heads of bundled spermatozoa. ×290.

the heads of bundles of spermatozoa (Fig. 10) whereas the heads on other bundles remained negative.

DISCUSSION

Our study demonstrates the presence of APGWa-immunoreactive substances in the CNS, the penis and the gonad of *Helix aspersa*. Whether the immunoreactive material is APGWa or cross-reacting substances cannot be determined by immunocytochemistry and should be determined by means of biochemical and/or molecular biological techniques. However, since APGWa had been demonstrated in distantly related molluscan species such as *Lymnaea stagnalis* (pulmonates) and *Fusinus ferrugineus* (prosobranchs), it may be assumed that APGWa occurs in *Helix aspersa*. Except the pedal ganglia, all ganglia contain immunoreactive neurons and most of the nerves possess immunoreactive fibres. This large distribution is consistent with the proposed role of APGWa in the muscular physiology: it has modulatory effects on the contractions of various molluscan muscles; depending on the muscles, it shows a stimulatory or an inhibitory action [1]. Concerning the penis, the peptide is probably involved in the contraction or the relaxation of the muscular layer during copulation. A comparable action of APGWa was also demonstrated in *Lymnaea stagnalis*, where APGWa antagonizes dopamine- and serotonin-induced contractions of the penis retractor muscle [2]. The origin of the positive fibres in the penis of *Helix* is unknown; according to previous descriptions, the axons of the penial nerve originate from the right pedal ganglion [8, 9]. As we never observed immunoreactive neurons in this ganglion, we can hypothesize that 1) a number of processes travel directly from the cerebral ganglia to the penis, 2) axons from different ganglia follow a lengthy way via the pedal ganglia to the penis.

The presence of APGWa-like substances in the right mesocerebral lobe is of great interest. Other peptides were immunocytochemically revealed in the mesocerebrum but they always show a bilateral distribution; this is the case for example for methionine-enkephalin [10], FMRFa [10] and somatostatin [11]. The asymmetrical occurrence of APGWa-like substance suggests that they might be responsible for the control of asymmetrical organs, in particular the reproductive organs which are located on the right side of the body. According to Chase [5], a mean number of 138 cells was counted in the right mesocerebrum of *Helix aspersa*. In our investigations, a high proportion of these cells (more than a quarter to a third) were APGWa-positive; their axons were traced to the right cerebro-pedal connective. This observation is in agreement with the results of dye-injections in mesocerebral neurons of the right side, demonstrating that the axons project almost without exception in the ipsilateral cerebro-pedal connective to the right pedal ganglion [5]. From electrophysiological studies [5, 12], we know that the mesocerebrum is a centre for coordinating the execution of mating. Firstly, electrical stimulations of the right mesocerebrum provoke large movements of the penis and the dart sac; these effects are mediated by axons that travel directly to the right pedal ganglion in which the motor-neurons lie [5]; secondly, the right mesocerebrum is responsible for the suppression of withdrawal responses during mating, by acting on the parietal command [12]; and thirdly, it can inhibit simultaneously a group of serotonergic neurons located in the pedal ganglia and capable of sensitizing the afferent excitation of the avoidance command cells [12]. Interestingly, also in *Lymnaea stagnalis* a group of serotonergic neurons in the right pedal ganglia, the so called pedal Ib cluster, are innervated by APGWa containing neurons in the right anterior lobus of the cerebral ganglia [4]. Our results suggest that APGWa-immunoreactive substances of the right mesocerebral participate at least at one of these functions but their mechanism of action remains to elucidate.

The presence of APGWa-immunoreactive substances on the head of spermatozoa is surprising. It is unlikely that the immunoreactive material could be the neuropeptide because we did not observe positive nervous fibres around the gonad; it consists more probably of immunologically related substances whose nature and significance must be investigated.

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